⑫ **EUROPEAN PATENT APPLICATION**

A request for correction of description page 9 + 10 in which the sequences refered to have been annatated with their sequence identification numbers has been filed pursuant to Rule 88 EPC. A decision on the request will be taken during the proceedings before the Examining Division (Guidelines for Examination in the EPO, A-V, 2.2).

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�54 Size markers for electrophoretic analysis of DNA.

㊗ A DNA marker ladder, useful in Southern blot hybridizations, is made up of pooled DNA restriction endonuclease digests, where each restriction digest contains at least one fragment complementary to a probe and at least one fragment not complementary to the probe. The regions of complementarity between the probe and the complementary fragments are double-stranded segments of the fragments. The ladder is characterized by an approximately even spacing of bands, resulting from choosing fragments having an logarithmic size distribution. Kits can incorporate this ladder and a probe, or means for making a probe, or a probe and a means for labelling a probe.

EP 0 466 404 A1

The present invention is in the field of molecular biology and specifically relates to the technique of gel electrophoresis of nucleic acid fragments.

A number of mixtures of nucleic acid fragments are commercially available that can be used as markers for determining the sizes of nucleic acid molecules of experimental int_rest. For example, Collaborative Research, Inc. (Lexington, MA) has sold a marker ladder ("Quik-Kit Size Markers", cat. no. 30013) that is a mixture of 12 bacteriophage *lambda* fragments. They are visualized by hybridization with two ^{32}P-labelled 12-nucleotide synthetic oligonucleotides, complementary to the left and right bacteriophage *cos* sites.

A large number of other DNA marker fragments are available from numerous suppliers. In every case, except the Collaborative markers, these marker fragments are restriction digests of several bacteriophage or plasmid DNAs. Every DNA fragment in the digests can then be visualized by hybridization to the same bacteriophage or plasmid DNAs.

Other DNA marker ladders often use collections of fragments that have a quasi-random size distribution. For example, the quasi-random size distribution may be made by a digest of a DNA, often *lambda* DNA, by a single restriction enzyme. Alternatively, the fragments may vary linearly with molecular weight, i.e. adjacent bands may differ by about 1000 base pairs (e.g. "1 Kb DNA Ladder", cat. no. 5615SA, BRL, Gaithersburg, MD). Bands in these linear ladders are not evenly spaced after electrophoresis, they are "compressed" in the "upper", higher molecular weight region of a gel. However some ladders have been constructed and sold that are logarithmically spaced ("GenePrint™ ", cat. no. DG1911, Promega, Madison, WI).

SUMMARY OF THE INVENTION

The drawback of conventional marker ladders is that the signal generated by each fragment is proportional to its length. As a result, levels of signal that allow visualization of small fragments (e.g. 500 base pairs (bp)) give too much signal in large fragments (e.g. 20 kbp) for optimal resolution. This drawback is overcome in the marker ladder of the present invention.

The invention consists of a "target DNA" and a "probe DNA". Target DNA is constructed by pooling several restriction endonuclease digests of a single DNA of known sequence. Each restriction endonuclease digest generates a number of DNA fragments, one of which contains a specific sequence "S". The restriction endonucleases and the sequence "S" are chosen so that the set of DNA fragments containing the same sequence "S" would give approximately a logarithmic distribution of lengths. In other words, when electrophoresed through a gel where nucleic acid fragments migrate as a logarithmic function of molecular weight, the marker fragments will be approximately evenly spaced and will leave no molecular weight range without a marker. When the pooled, digested DNA is electrophoresed in a gel matrix, a ladder of fragments is generated containing sequence "S", with approximately equal spacing between them.

The probe DNA is complementary to sequence "S", and therefore can be bound specifically to sequence "S" by nucleic acid hybridization. When the probe DNA is labelled (for example, with radioactive phosphorus, biotin, or alkaline phosphatase) it allows visualization of the DNA fragments containing sequence "S".

The present invention preferably utilizes internal labelling sites, thus allowing both ends of the DNA fragment to be altered by restriction endonuclease cleavage. Therefor, a greater variety of DNA fragment sizes can be generated.

The present invention is expected to be useful to research laboratories employing DNA or RNA analysis techniques and it is especially useful to laboratories and law enforcement agencies using DNA analysis to identify individuals.

Preferred embodiments of the invention will now be described with reference to the drawing, in which:

FIGURE 1 is a schematic, scale drawing of the how the first and second molecular marker kits would migrate on an electrophoretic gel. The positions were calculated by assuming that relative mobilities are a linear function of the logarithm of the length of the fragment in base pairs (bp). The length of each band in bp is indicated to the left of the band.

The present invention is a DNA size marker system, preferably a DNA marker ladder, having pooled DNA restriction endonuclease digests. By the term "DNA marker ladder" is meant DNA fragments of varying sizes containing the sequence "S" that when electrophoresed through a gel matrix migrate with approximately equal spacing between them. "Equal spacing" may refer either to the physical location on a gel after electrophoresis (e.g. bands about 0.5 cm. apart) or to the size being marked (e.g. bands differing in size by 1,000 bp). Each restriction digest contains at least one DNA fragment having an "S" sequence complementary to a probe and one or more other DNA fragments not complementary to the probe. The same probe is thus used for all restriction digests. The region of complementarity between the probe and the first DNA fragment of each digest is a double-stranded segment of the first fragment.

The number of restriction digests pooled is at least 5, preferably at least 10, more preferably at least 15,

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yet more preferably at least 20, and most preferably at least 25. In the present invention, the largest target fragment is at least 10-fold, preferably 14-fold, and most preferably 17-fold, longer than the smallest target fragment.

In some embodiments, target fragments most similar in size differ in length by defined amounts. As defined herein, the "measure", M, of the difference in size is herein calculated by the formula $M = \log_{10}(U)-\log_{10}(L)$, where U and L are the respective lengths in bp of the upper and lower of the two adjacent bands being compared. This equation is equivalent to $10^M = U/L$. As a means of illustration, Table 1 shows the relationship between M, U, and L, (U and L are in bp) with the latter being held constant at 1,000 bp. Note that if U and L are both changed by the same factor or multiple, M remains constant. For example, bands of 1,059 bp and 1,000 bp and bands of 530 bp and 500 bp both differ in size by measures of 0.025. Preferably, target fragment pairs most similar in size differ in size by no more than a measure of about 0.1 (e.g bands of 1,259 bp and 1,000 bp), and, most preferably, by no more than a measure of about 0.075 (e.g bands of 1,188 bp and 1,000 bp). In other words, bands that after gel electrophoresis and Southern blotting would be adjacent to each other differ in size by no more than a measure of about 0.1. As exemplified herein, the target fragment pairs most similar in size differ in size by at least a measure of about 0.025 (e.g. bands of 1,059 bp and 1,000 bp).

Preferably, the target fragments all anneal to a single probe sequence or its complement. More than one molecular species may be in the probe, provided that each digest contains at least one fragment that can anneal to a probe molecule and at least one fragment that cannot anneal to a probe molecule. Although not meant to be limiting, as exemplified herein, the target fragments are derived from bacteriophage *lambda*. As also exemplified herein, the target fragments may be detected with a probe having sequence present in or a sequence complementary to a sequence present in nucleotides 33,783 to 34,212 of bacteriophage *lambda.*

The present invention may further be included in a kit having, in addition to the target fragments, a probe nucleic acid complementary to target DNA fragments. As exemplified herein, the sequence of the probe is present in or is complementary to a sequence present in nucleotides 33,783 to 34,212 of bacteriophage *lambda.*

The kit may further include an enzyme capable of radioactively labelling the probe, e.g. polynucleotide kinase or the Klenow fragment of *E. coli* DNA polymerase I.

Preferably, the target DNA is constructed from a single bacteriophage or plasmid. The target DNA preferably consists of at least 10 restriction endonuclease digests of that target DNA. Each restriction digest of the target DNA creates one fragment complementary to the probe DNA, and the lengths of these fragments may be distributed in a logarithmic array.

Preferably, the probe DNA is supplied as a pair of synthetic oligonucleotides. Each of the probe oligonucleotides is preferably at least 20 nucleotides in length and are complementary to each other for 15 to 30 base pairs at their 3'-ends. These oligonucleotides can then be labelled by incorporation of labelled nucleotides in a chain extension reaction, with each oligonucleotide serving as a primer and using the other as a template in the chain extension reaction. As an illustration, in the following arrangement the upper and lower case letters are complements of each other:

<div align="center">

5'abc...lmnopq3'

3'OPQRST...XYZ5'

</div>

After chain extension with a labelled nucleotide, here indicated by <u>underlining</u>, the oligonucleotides will have the following structure:

<div align="center">

5'abc...lmnop<u>qrst...xyz</u>3'

3'<u>ABC...LMN</u>OPQRST...XYZ5'

</div>

This structure can then be separated to form two probes labelled at their 3'-ends: 5'abc...lmnop<u>qrst...xyz</u>3' and 5'ZYX...TSRQP<u>ONML...CBA</u>3'.

The probe may be labelled with a radioisotope (e.g. ^{3}H, ^{32}P, ^{35}S, or ^{125}I), a ligand (e.g. biotin), a hapten (e.g. dinitrophenol, fluorescein), or an enzyme (e.g. alkaline phosphatase, β-galactosidase, horseradish peroxidase, microperoxidase), or any other suitable labelling method known to or discovered by the art. The choice of labelling method will generally depend on the chosen method for detecting the experimental sample for which the marker kit is serving as a molecular weight standard.

A DNA marker kit of the present invention also include a means for making a probe, instead of just a means for added labelled nucleotides, e.g with DNA polymerase, or another labelled entity, e.g. ^{32}pO$_4$ and kinase. This means may be a means for making an RNA probe. They means for making a probe may include being probe sequences under control of a promoter (i.e. a means-DNA). The kit could also include an RNA polymerase cap-

able of initiating transcription from the promoter and transcribing probe sequences of the means-DNA. Examples of such means-DNAs and RNA polymerases are well known in the art. For instance, DNA sequences downstream from SP6 promoters are commonly transcribed *in vitro* by SP6 RNA polymerase and sequences downstream from T7 promoters ar_ commonly transcribed *in vitro* by T7 RNA polymerase.

5   In an actual gel electrophoresis, the bands may not be spaced exactly as shown in Figure 1 due to well known phenomena concerning mobility of very large and very small fragments, sample loading effects, and inhomogeneities in the gel. With the use of the present invention, these effects can be detected more readily. Indeed, due to the way that DNA fragments run in 1.0% agarose gels, the largest (e.g. above 10 kbp) target fragments of the exemplified kits will appear more evenly spaced than as illustrated in Figure 1.

10   The DNA marker fragments should be hybridized with the probe, with the fragments which bind probe molecules being the fragments detected. When the total DNA of these ladder kits is inspected by non-specific, sequence-independent staining, e.g. with ethidium bromide, the ladder DNA may appear as a "smear" due to the multitude of fragments.

    Although specific restriction endonucleases are recited in the Examples and the Claims, it will be recog-

15 nized that isoschizomers, i.e. enzymes that have the same recognition sequence but cut in a different fashion, can be substituted and the same result will be achieved.

    The following examples illustrate but do not limit the invention.

EXAMPLES

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Example 1: Common Materials and Methods

    E. coli bacteriophage *lambda* DNA (clind 1, ts857, Sam 7) was the source of all target DNAs.

    The probe DNA for either of the ladders exemplified herein may consist of any DNA from between nuc-

25 leotides 33,783 and 34,212 of that *lambda* DNA. Oligonucleotides were synthesized using standard phosphoramidite chemistry well known to the art.

    To make a restriction digest, *lambda* DNA was digested with one or two restriction endonucleases. The enzymes used for individual digests are indicated in Tables 2 and 3. Digestions were performed under standard conditions, generally according to the instructions of the enzyme's manufacturer. Restriction digests were

30 pooled after digestion.

Example 2: First Marker Kit

    In the first ladder, the target DNA consisted of pooled equal amounts of 31 different restriction digests of

35 phage *lambda* DNA. The probe DNA was a 26-base oligonucleotide having a sequence of

$$5\,'GCGACATTGCTCCGTGTATTCACTCG3\,'$$

which is complementary to nucleotides 34,000 to 34,025 of the standard *lambda* DNA map. This oligonucleotide

40 was labelled at its 5'-end by T4 polynucleotide kinase and [*lambda*-^{32}P]-ATP (BRL cat. no. 8060SA, Life Technologies, Inc., Gaithersburg, MD). Hybridization of ^{32}P-labelled probe DNA to a Southern blot of the target DNA revealed bands of the expected pattern (Figure 1). The restriction endonuclease digestions used, the sizes of the fragments generated thereby, the *lambda* sequence coordinates thereof, and the measures of the size differences between adjacent bands are listed in Table 2.

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Example 3: Second Marker Kit

    This first kit was improved in three ways. The first improvement was to change the probe DNA such that (a) it could easily be labelled with DNA polymerase as well as polynucleotide kinase, and (b) it would remain

50 hybridized to the Southern blot even when washed at high temperature (65°C) and low salt concentration (0.015 M NaCl). This was achieved by utilizing two 70-base, synthetic oligonucleotides that were complementary to opposite strands of *lambda* DNA, and also complementary to one another for 15 bases at their 3'-termini. The two oligonucleotides were as follows:

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5'AGGCCACTATCAGGCAGCTTTGTTGTTCTGTTTACCAAGTTCTCTGGCAATCATT<u>GCCGTCGTTCGTATT</u>3'

5'AGCCTGAAGAAATGTTTCCTGTAATGGAAGATGGGAAATATGTCGATAAATGGGC<u>AATACGAACGACGGC</u>3'

The underlined segments are complementary to each other. The first oligonucleotide is encoded by sequences from coordinates 34,078 (5'-end) to 34,147 (3'-end) and the second oligonucleotide is encoded by sequences from 34,133 (3'-end) to 34,202 (5'-end) on the standard *lambda* map. These oligonucleotides were mixed together with each other and the Klenow fragment of *E. coli* DNA polymerase I and four deoxynucleotide triphos-
5 phates, one of which was α-^{32}P-labelled. The polymerase extended each oligonucleotide using the other as a template and produced two α-^{32}P-labelled, complementary oligonucleotides. This new probe hybridizes to the same target fragments as the previous probe. A mixture of the new 70-mers was labelled with the large fragment of *E. coli* DNA polymerase I and hybridized to a Southern blot of the target DNA. The second improvement was to change the target DNA to give a more linear spacing on the Southern blot.
10 The third improvement was to increase the amounts, i.e. relative copy number or the dosage, of the target DNA for the largest and smallest bands. Large DNA fragments blot inefficiently. As is well known in the art, small fragments are retained on membranes poorly during hybridization. Therefore, the signal from large DNA fragments and small DNA fragments tends to be less than the signal from bands in the middle range. This improvement compensated for that effect.
15 Hybridization of ^{32}P-labelled probe DNA to a Southern blot of the target DNA revealed bands of the expected pattern (Figure 1). The restriction endonuclease digestions and dosage used, the sizes of the fragments generated thereby, the *lambda* sequence coordinates thereof, and measures of the size differences between adjacent bands are listed in Table 3.

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Table 1:   Examples of Relationships between the Measure
           of the Difference in Size and Sizes of
           Fragments.

M	U	L
0.0	1,000	1,000
0.025	1,059	1,000
0.05	1,122	1,000
0.075	1,188	1,000
0.1	1,259	1,000
0.15	1,413	1,000
0.2	1,585	1,000
0.3	1,995	1,000
0.5	3,162	1,000
0.7	5,012	1,000
1.0	10,000	1,000

$M = \log_{10}(U) - \log_{10}(L)$ = Measure of the difference in size.

U = Size in bp of the upper band in a comparison.

L = Size in bp of the lower band in a comparison, held constant at 1,000 bp.

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Table 2: DNA Analysis Marker Ladder Target DNA
Fragments, First Kit

Enzyme(s)	Size	Diff.	Lambda Coordinates	
			Left	Right
Xba I *	23,994	0.204	24,508	48,502
Xho I	15,004	0.127	33,498	48,502
Xba I/Bgl II *	11,203	0.075	24,508	35,711
Hind III	9,416	0.056	27,479	36,895
Sma I	8,271	0.047	31,619	39,890
EcoR I	7,421	0.061	31,747	39,168
Ava II	6,442	0.041	32,562	39,004
Hae II	5,861	0.034	28,859	34,720
EcoR V/Ava II	5,415	0.060	33,589	39,004
Ava I	4,716	0.067	33,498	38,214
Bgl I/BstE II *	4,045	0.026	32,329	36,374
Ava II/BstE II	3,812	0.025	32,562	36,374
Dra I *	3,599	0.065	32,705	36,304
Sma I/Hae II	3,101	0.033	31,619	34,720
Xho I/BstE II	2,876	0.036	33,498	36,374
Nci I	2,650	0.037	33,158	35,808
Nde I	2,433	0.026	33,680	36,113
Msp I *	2,293	0.056	33,157	35,450
Hinc II	2,015	0.035	33,246	35,261
EcoR V/Msp I	1,861	0.023	33,589	35,450
Xho I/Hinc II *	1,763	0.051	33,498	35,261
Rsa I	1,568	0.040	32,868	34,436
Ssp I	1,431	0.028	33,572	35,003
Msp I/BamH I *	1,342	0.057	33,157	34,499
Sau3A I	1,176	0.024	33,323	34,499
Cla I *	1,112	0.087	33,585	34,697
EcoR V/BamH I	910	0.033	33,589	34,499
Hinf I *	844	0.064	33,783	34,627
EcoR V/Cvn I *	730	0.048	33,589	34,319
Hinf I/Rsa	653	0.094	33,783	34,436
Nsi	526	-----	33,686	34,212

Diff. = The difference, M, in size between the band and the band immediately below, calculated by the formula, $M = \log_{10}(U) - \log_{10}(L)$, where U and L are the lengths in bp of the upper and lower, respectively, of the two bands being compared.

* indicates enzyme combinations used in the first ladder but not used in the second ladder.

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Table 3: DNA Analysis Marker Ladder Target DNA Fragments, Second Kit

Enzyme(s)	Size	Diff.	Lambda Coordinates		
			Left	Right	Dose
Sst I *	22,621	0.178	25,881	48,502	3
Xho I	15,004	0.100	33,498	48,502	3
Nco I/Bgl I *	11,919	0.102	32,329	44,248	3
Hind III	9,416	0.056	27,479	36,895	3
Sma I	8,271	0.047	31,619	39,890	3
EcoR I	7,421	0.061	31,747	39,168	3
Ava II	6,442	0.041	32,562	39,004	3
Hae II	5,861	0.034	28,859	34,720	1
EcoR V/Ava II	5,415	0.060	33,589	39,004	1
Ava I	4,716	0.037	33,498	38,214	1
Ava II/Hind III *	4,333	0.056	32,562	36,895	1
Ava II/BstE II	3,812	0.050	32,562	36,374	1
Xho I/Hind III *	3,397	0.040	33,498	36,895	1
Sma I/Hae II	3,101	0.033	31,619	34,720	1
Xho I/BstE II	2,876	0.036	33,498	36,374	1
Nci I	2,650	0.037	33,158	35,808	1
Nde I	2,433	0.041	33,680	36,113	1
Xho I/Bgl II *	2,213	0.041	33,498	35,711	1
Hinc II	2,015	0.035	33,246	35,261	1
EcoR V/Msp I	1,861	0.047	33,589	35,450	1
EcoR V/Hinc II *	1,672	0.028	33,589	35,261	1
Rsa I	1,568	0.040	32,868	34,436	1
Ssp I	1,431	0.046	33,572	35,003	1
Tha I/Rsa I *	1,287	0.039	33,149	34,436	1
Sau3A I	1,176	0.073	33,323	34,499	1
Cfo I *	993	0.038	33,726	34,719	1
EcoR V/BamH I	910	0.065	33,589	34,499	1
Dde I *	784	0.079	33,535	34,319	3
Hinf I/Rsa I	653	0.094	33,783	34,436	3
Nsi I	526	-----	33,686	34,212	3

Diff. = The difference, M, in size between the band and the band immediately below, calculated by the formula $M = \log_{10}(U) - \log_{10}(L)$, where U and L are the lengths in bp of the upper and lower, respectively, of the two bands being compared.

* indicates enzyme combinations used in the second ladder but not used in the first ladder.

Dose refers to the relative amounts of each restriction digest.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following Claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: Life Technologies, Inc
        (B) STREET: 8717 Grovemont Circle, PO Box 9418
        (C) CITY: Gaithersburg
        (D) STATE: Maryland
        (E) COUNTRY: USA
        (F) POSTAL CODE (ZIP): 20898

    (ii) TITLE OF INVENTION: Size Markers for Electrophoretic Analysis of
        DNA

    (iii) NUMBER OF SEQUENCES: 3

    (iv) COMPUTER READABLE FORM:
        Not Applicable

    (v) CURRENT APPLICATION DATA:
        APPLICATION NUMBER: EP 91306104.0

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGACATTGC TCCGTGTATT CACTCG   26

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 70 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGGCCACTAT CAGGCAGCTT TGTTGTTCTG TTTACCAAGT TCTCTGGCAA TCATTGCCGT   60

CGTTCGTATT   70

(2) INFORMATION FOR SEQ ID NO:3:

9

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(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 70 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGCCTGAAGA AATGTTTCCT GTAATGGAAG ATGGGAAATA TGTCGATAAA TGGGCAATAC      60

GAACGACGGC                                                            70
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Claims

1. A DNA marker system comprising at least 5 DNA restriction endonuclease digests pooled together, wherein

   (1) a DNA restriction endonuclease digest is a collection of DNA fragments resulting from digestion of a DNA by one or more restriction endonucleases,

   (2) each restriction digest contains a first DNA fragment complementary to a probe,

   (3) each restriction digest contains a second DNA fragment not complementary to the probe, and

   (4) the probe is the same probe for all the restriction digests, and

   (5) the region of complementarity between the probe and the first DNA fragment of each digest is a double-stranded segment of the first fragment.

2. A system as claimed in claim 1, comprising, in increasing order of preference, at least 10, 15, 20 or 25 DNA restriction endonuclease digests pooled together.

3. A system as claimed in claim 1 or 2, wherein adjacent target fragment pairs differ in size by no more than a measure of about 0.1, and preferably by no more than a measure of about 0.075.

4. A system as claimed in claim 1, 2 or 3, wherein adjacent target fragment pairs differ in size by at least a measure of about 0.025.

5. A system as claimed in any one of claims 1 to 4, wherein the largest target fragment is, in increasing order of preference, at least 10-fold, 14-fold or 17-fold longer than the smallest target fragment.

6. A system as claimed in any one of claims 1 to 5, wherein the target fragments are derived from bacteriophage *lambda*.

7. A system as claimed in claim 6, wherein the target fragments may be detected with a probe having sequence present in or a sequence complementary to a sequence present in nucleotides 33,783 to 34,212 of bacteriophage *lambda*.

8. A system as claimed in claim 6 or 7, wherein the target fragments include at least 10 fragments and are chosen from a group of DNA fragments having sizes and ends of 11,203 bp *Xba* I/*Bgl* II, 9,416 bp *Hind* III, 8,271 bp *Sma* I, 7,421 bp *EcoR* I, 6,442 bp *Ava* II, 5,861 bp *Hae* II, 5,415 bp *EcoR* V/*Ava* II, 4,716 bp *Ava* I, 4,333 bp *Ava* II/*Hind* III, 4,045 bp *Bgl* I/*BstE* II, 3,812 bp *Ava* II/*BstE* II, 3,599 bp *Dra* I, 3,397 bp *Xho* I/*Hind* III, 3,101 bp *Sma* I/*Hae* II, 2,876 bp *Xho* I/*BstE* II, 2,650 bp *Nic* I, 2,433 bp *Nde* I, 2,293 bp *Msp* I, 2,213 bp *Xho* I/*Bgl* II, 2,015 bp *Hinc* II, 1,861 bp *EcoR* V/*Msp* I, 1,763 bp *Xho* I/*Hinc* II, 1,672 bp *EoR* V/*Hinc* II, 1,568 bp *Rsa* I, 1,431 bp *Ssp* I, 1,342 bp *Msp* I/*BamH* I, 1,287 bp *Tha* I/*Rsa* I, 1,176 bp *Sau3A* I, 1,112 bp *Cla* I, 993 bp *Cfo* I, 910 bp *EcoR* V/*BamH* I, 844 bp *Hinf* I, 784 bp *Dde* I, 730 bp *EcoR* V/*Cvn*

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I, and 653 bp *Hinf* I/*Rsa* I.

9. A system as claimed in claim 6, 7 or 8, wherein the target fragments include at least 15 fragments and are chosen from a group of DNA fragments having sizes and ends of 11,203 bp *Xba* I/*Bgl* II, 9,416 bp *Hind* III, 8,271 bp *Sma* I, 7,421 bp *EcoR* I, 6,442 bp *Ava* II, 5,861 bp *Hae* II, 5,415 bp *EcoR* V/*Ava* II, 4,716 bp *Ava* I, 4,333 bp *Ava* II/*Hind* III, 4,045 bp *Bgl* I/*BstE* II, 3,812 bp *Ava* II/*BstE* II, 3,599 bp *Dra* I, 3,397 bp *Xho* I/*Hind* III, 3,101 bp *Sma* I/*Hae* II, 2,876 bp *Xho* I/*BstE* II, 2,650 bp *Nci* I, 2,433 bp *Nde* I, 2,293 bp *Msp* I, 2,213bp *Xho* I/*Bgl* II, 2,015 bp *Hinc* II, 1,861 bp *EcoR* V/*Msp* I, 1,763 bp *Xho* I/*Hinc* II, 1,672 bp *EcoR* V/*Hinc* II, 1,568 bp *Rsa* I, 1,431 bp *Ssp* I, 1,342 bp *Msp* I/*BamH* I, 1,287 bp *Tha* I/*Rsa* I, 1,176 bp *Sau3A* I, 1,112 bp *Cla* I, 993 bp *Cfo* I, 910 bp *EcoR* V/*BamH* I, 844 bp *Hinf* I, 784 bp *Dde* I, 730 bp *EcoR* V/*Cvn* I, and 653 bp *Hinf* I/*Rsa* I.

10. A system as claimed in any one of claims 6 to 9, wherein the target fragments comprise at least 20 fragments and are chosen from a group of DNA fragments having sizes and ends of 11,203 bp *Xba* I/*Bgl* II, 9,416 bp *Hind* III, 8,271 bp *Sma* I, 7,421 bp *EcoR* I, 6,442 bp *Ava* II, 5,861 bp *Hae* II, 5,415 bp *EcoR* V/*Ava* II, 4,716 bp *Ava* I, 4,333 bp *Ava* II/*Hind* III, 4,045 bp *Bgl* I/*BstE* II, 3,812 bp *Ava* II/*BstE* II, 3,599 bp *Dra* I, 3,397 bp *Xho* I/*Hind* III, 3,101 bp *Sma* I/*Hae* II, 2,876 bp *Xho* I/*BstE* II, 2,650 bp *Nci* I, 2,433 bp *Nde* I, 2,293 bp *Msp* I, 2,213 bp *Xho* I/*Bgl* II, 2,015 bp *Hinc* II, 1,861 bp *EcoR* V/*Msp* I, 1,763 bp *Xho* I/*Hinc* II, 1,672 bp *EcoR* V/*Hinc* II, 1,568 bp *Rsa* I, 1,431 bp *Ssp* I, 1,342 bp *Msp* I/*BamH* I, 1,287 bp *Tha* I/*Rsa* I, 1,176 bp *Sau3A* I, 1,112 bp *Cla* I, 993 bp *Cfo* I, 910 bp *EcoR* V/*BamH* I, 844 bp *Hinf* I, 784 bp *Dde* I, 730 bp *EcoR* V/*Cvn* I, and 653 bp *Hinf* I/*Rsa* I.

11. A system as claimed in any one of claims 6 to 10, wherein the target fragments comprise at least 25 fragments and are chosen from a group of DNA fragments having sizes and ends of 11,203 bp *Xba* I/*Bgl* II, 9,416 bp *Hind* III, 8,271 bp *Sma* I, 7,421 bp *EcoR* I, 6,442 bp *Ava* II, 5,861 bp *Hae* II, 5,415 bp *EcoR* V/*Ava* II, 4,716 bp *Ava* I, 4,333 bp *Ava* II/*Hind* III, 4,045 bp *Bgl* I/*BsEII*, 3,812 bp *Ava* II/*BstE* II, 3,599 bp *Dra* I, 3,397 bp *Xho* I/*Hind* III, 3,101 bp *Sma* I/*Hae* II, 2,876 bp *Xho* I/*BstE* II, 2,650 bp *Nci* I, 2,433 bp *Nde* I, 2,293 bp *Msp* I, 2,213 bp *Xho* I/*Bgl* II, 2,015 bp *Hinc* II, 1,861 bp *EcoR* V/*Msp* I, 1,763 bp *Xho* I/*Hinc* II, 1,672 bp *EcoR* V/*Hinc* II, 1,568 bp *Rsa* I, 1,431 bp *Ssp* I, 1,342 bp *Msp* I/*BamH* I, 1,287 bp *Tha* I/*Rsa* I, 1,176 bp *Sau3A* I, 1,112 bp *Cla* I, 993 bp *Cfo* I, 910 bp *EcoR* V/*BamH* I, 844 bp *Hinf* I, 784 bp *Dde* I, 730 bp *EcoR* V/*Cvn* I, and 653 bp *Hinf* I/*Rsa* I.

12. A system as claimed in any one of claims 6 to 11, wherein the target fragments comprise at least 25 fragments and are chosen from a group of DNA fragments having sizes and ends of 9,416 bp *Hind* III, 8,271 bp *Sma* I, 7,421 bp *EcoR* I, 6,442 bp *Ava* II, 5,861 bp *Hae* II, 5,415 bp *EcoR* V/*Ava* II, 4,716 bp *Ava* I, 4,333 bp *Ava* II/*Hind* III, 3,812 bp *Ava* II/*BstE* II, 3,397 bp *Xho* I/*Hind* III, 3,101 bp *Sma* I/*Hae* II, 2,876 bp *Xho* I/*BstE* II, 2,650 bp *Nci* I, 2,433 bp *Nde* I, 2,213 bp *Xho* I/*Bgl* II, 2,015 bp *Hinc* II, 1,861 bp *EcoR* V/*Msp* I, 1,672 bp *EcoR* V/*Hinc* II, 1,568 bp *Rsa* I, 1,431 bp *Ssp* I, 1,287 bp *Tha* I/*Rsa* I, 1,176 bp *Sau3A* I, 993 bp *Cfo* I, 910 bp *EcoR* V/*BamH* I, 784 bp *Dde* I, and 653 bp *Hinf* I/*Rsa* I.

13. A system as claimed in any one of claims 6 to 12, wherein the target fragments have sizes and ends of 22,621 bp *Sst* I, 15,004 bp *Xho* I, 11,919 bp *Nco* I/*Bgl* I, 9,416 bp *Hind* III, 8,271 bp *Sma* I, 7,421 bp *EcoR* I, 6,442 bp *Ava* II, 5,861 bp *Hae* II, 5,415 bp *EcoR* V/*Ava* II, 4,716 bp *Ava* I, 4,333 bp *Ava* II/*Hind* III, 3,812 bp *Ava* II/*BstE* II, 3,397 bp *Xho* I/*Hind* III, 3,101 bp *Sma* I/*Hae* II, 2,876 bp *Xho* I/*BstE* II, 2,650 bp *Nci* I, 2,433 bp *Nde* I, 2,213 bp *Xho* I/*Bgl* II, 2,015 bp *Hinc* II, 1,861 bp *EcoR* V/*Msp* I, 1,672 bp *EcoR* V/*Hinc* II, 1,568 bp *Rsa* I, 1,431 bp *Ssp* I, 1,287 bp *Tha* I/*Rsa* I, 1,176 bp *Sau3A* I, 993 bp *Cfo* I, 910 bp *EcoR* V/*BamH* I, 784 bp *Dde* I, 653 bp *Hinf* I/*Rsa* I, and 526 bp *Nsi* I.

14. A system as claimed in any one of claims 1 to 13, wherein relative quantities of each fragment is such that in a Southern blot hybridization observed band intensities are uniform within a factor of 2.

15. A DNA marker kit comprising
   (a) A DNA marker system comprising at least 5 DNA restriction endonuclease digests pooled together, wherein
      (1) a DNA restriction endonuclease digest is a collection of DNA fragments resulting from digestion of a DNA by one or more restriction endonucleases,
      (2) each restriction digest contains a first DNA fragment complementary to a probe,
      3) each restriction digest contains a second DNA fragment not complementary to the probe, and

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(4) the probe is the same probe for all the restriction digests, and

(5) the region of complementarity between the probe and the first DNA fragment of each digest is a double-stranded segment of the first fragment, and

a first probe nucleic acid complementary to first target DNA fragments.

16. A kit as claimed in claim 15, further comprising a second probe nucleic acid complementary to target DNA fragments, wherein the first probe and the second probe are DNA, are complementary to each other at their 3'-ends, and are not complementary to each other at their 5'-ends.

17. A kit as claimed in claim 15 or 16, wherein the sequence of the first probe is present in or is complementary to a sequence present in nucleotides 33, 783 to 34, 212 of bacteriophage *lambda.*

18. A kit as claimed in claim 15, 16 or 17, further comprising an enzyme capable of labelling the probe, for example radioactively.

19. A kit as claimed in claim 18, wherein the enzyme is a DNA polymerase, such as he Klenow fragment of *E. coli* DNA polymerase I, or polynucleotide kinase.

20. A DNA marker kit comprising a DNA marker system as claimed in any one of claims 1 to 14 and means for making a probe.

21. A kit as claimed in claim 20, wherein the means for making a probe is means for making an RNA probe.

22. A kit as claimed in claim 21, wherein the means for making a probe comprises

(a) a means-DNA, wherein the means-DNA comprises probe sequences under control of a promoter, and

(b) an RNA polymerase capable of initiating transcription from the promoter and transcribing probe sequences of the means-DNA.

23. A process for preparing a system as claimed in any one of claims 1 to 14, the process comprising digesting DNA with endonuclease(s) and/or pooling endonuclease digests.

24. A process for preparing a kit as claimed in any one of claims 15 to 22, the process comprising providing the components in association but not admixture.

FIGURE 1

First Kit	Second Kit
Size Position	Size Position

First Kit sizes:
23994
15004
11203
9416
8271
7421
6442
5861
5415
4716
4045
3812
3599
3101
2876
2650
2433
2293
2015
1861
1763
1568
1431
1342
1176
1112
910
844
730
653
526

Second Kit sizes:
22621
15004
11919
9416
8271
7421
6442
5861
5415
4716
4333
3812
3397
3101
2876
2650
2433
2213
2015
1861
1672
1568
1431
1287
1176
993
910
784
653
526

13

European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 30 6104

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-0 357 028 (BIOTEST AG) * Column 9, line 44 - column 10, line 2 * ---	1,5	C 12 Q 1/68 C 12 Q 1/70 C 12 Q 1/34 C 07 H 21/04 // C 12 P 19/34 G 01 N 27/26
A	CHEMICAL ABSTRACTS, vol. 105, no. 11, 15th September 1986, page 187, abstract no. 92470r, Columbus, Ohio, US; A. BERNARDS et al.: "Pulsed field gradient electrophoresis of DNA digested in agarose allows the sizing of the large duplication unit of a surface antigen gene in trypanosomes", & GENE 1986, 42(3), 313-22 * Abstract * ---	1,5	
A	CHEMICAL ABSTRACTS, vol. 107, no. 5, 3rd August 1987, page 175, abstract no. 34093a, Columbus, Ohio, US; J. HUANG et al.: "Restriction endonuclease analysis of granulosis virus DNA of Agrotis exclamationis Linnaeus", & KUNCHONGXUE YANJIU JIKAN 1985, 5, 155-62 * Abstract * ---	1,5	**TECHNICAL FIELDS SEARCHED (Int. Cl.5)** C 12 Q C 12 N G 01 N
A	CHEMICAL ABSTRACTS, vol. 112, no. 25, 18th June 1990, page 172, abstract no. 230760h, Columbus, Ohio, US; C.P. JONES et al.: "Separation of yeast chromosomes in the megabase range suitable as size markers for pulsed-field gel electrophoresis", & TECHNIQUE (PHILADELPHIA) 1989, 1(2), 90-5 * Abstract * --- -/-	1,5	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	18-10-1991	OSBORNE H.H.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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T : theory or principle underlying the invention
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EUROPEAN SEARCH REPORT

Page 2

Application Number

EP 91 30 6104

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	D. RICKWOOD et al.: "Gel electrophoresis of nucleic acids, a practical approach", published in the "Practical approaches in biochemistry series", IRL Press, Oxford, GB; APPENDIX I: "Nucleic acid molecular weight markers" * Pages 227-232 *	1,5	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	18-10-1991	OSBORNE H.H.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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FIGURE 1

	First Kit		Second Kit
	Size Position		Size Position

First Kit

23994 —————

15004 —————

11203 —————

9416 —————

8271 —————
7421 —————

6442 —————
5861 —————
5415 —————

4716 —————

6045
3812
3599

3101
2876
2650
2433
2275

2015
1861
1763

1568

1431
1342

1176
1112

910
844

750

653

526 —————

Second Kit

22621 —————

15004 —————

11919 —————

9416 —————

8271 —————
7421 —————

6442 —————
5861 —————
5415 —————

4716 —————
4533

3812

3397
3101
2876
2650
2433
2213

2015
1861

1672
1568

1431

1287
1176

993
910

764

653

526 —————